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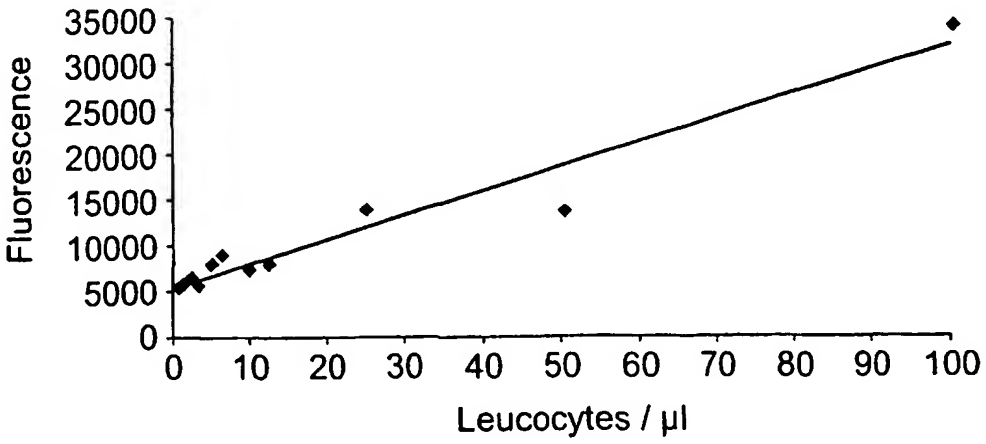
(54) Title: CELL COUNTING METHOD AND KIT



(57) Abstract: There is provided a method for quantifying the number of target cells in a sample which comrises: a) applying the sample to a substrate, a surface of which is provided with a plurality of binding moieties each capable of binding specifically to the target cell so as to retain said target cells bound to said substrate surface, there being sufficient of said binding moieties to retain substantially all of the said target cells in the sample; b) washing the substrate and captured target cells to remove any extraneous non-specifically bound matter; c) lysing the retained target cells; and d) quantifying at least one component of the retained target cells to provide an indication of the number of retained target cells.

WO 01/11362 A1

CELL COUNTING METHOD AND KIT

This invention relates to a method for quantifying the number of target cells in a sample, and a kit for quantifying the number of target cells in a sample using such a method.

There are a number of applications in which it is desirable to quantify the total number of cells or the number of cells of a particular type in a sample where the cells are at a low concentration (for example, in the range of 0.001-100 cells μL^{-1}).

For example, in order to comply with UK guidelines for leucodepleted blood products, it is necessary to determine the level of residual donor leucocyte contamination in therapeutic components. After leucodepletion, the leucocyte concentration in the blood product is commonly below 20 cells μL^{-1},

Currently available technologies for cell counting include counting the cells by eye using a haemocytometer and a light microscope, or automated counting by flow cytometry.

Counting by eye is labourious, time-consuming and, since it is open to an element of human error, frequently inaccurate. Flow cytometry is also time-consuming, since each sample has to be analysed individually. Also, flow cytometry machines are very expensive to buy and run, and require continual maintenance. Finally, the results obtained can vary from one machine to another, so even though the counting process is automated, it is not truly reproducible.

Another problem with both of the currently used methods mentioned above is that in the desired cell concentration range, the methods are operating at the lower limit of their sensitivities, which inevitably makes the results obtained less accurate and

reproducible. It is possible to include a step to increase the concentration of the cells prior to counting (for example by using a centrifugation step) but this can have the effect of making the processes more complicated, time consuming and inaccurate.

Another application in which it is desirable to quantify the number of cells of a particular type in a sample where the cells are at a low concentration is in the analysis of fetal rhesus-positive red-blood cells in rhesus-negative mothers. By way of brief background, in 1939, Philip Levine and Rufus Stevenson described the passage of Rhesus antibodies from the mother to the fetus, resulting in haemolytic disease of the newborn (HDN). Prior to the development of treatment, 1 in 200 pregnant mothers developed Rhesus antibodies. A fifth of these mothers had stillborn infants during their first pregnancy and the rate of stillbirths increased with subsequent pregnancies. The transplacental passage of fetal Rhesus D positive (D+) red blood cells (RBCs) into a Rhesus D negative (D-) woman before or after delivery results in the formation of anti-D antibodies. The anti-D IgG is then transported back across the placenta into the fetus, resulting in haemolysis of fetal RBCs. Anti-D mediated haemolytic disease can affect both the current pregnancy and subsequent pregnancies with a D+ fetus.

The production of maternal anti-D may be prevented by administration of prophylactic anti-D within 72 hours of the fetomaternal haemorrhage (FMH). Prophylactic anti-D is given to all D- women after delivery of a D+ neonate, or after any other event likely to cause a FMH. The minimum dose (500-1500 IU of IgG) is sufficient to clear 4-12mL of D+ cells, calculated at a ratio of 125 IU (25μg) to 1mL of cells. The volume of the FMH must be determined to calculate

whether additional anti-D is required. It is currently quantitated using either of the Kleihauer-Betke acid elution technique or by flow cytometry.

According to a first aspect of the invention there is provided a method for quantifying the number of target cells in a sample which comprises;

(a) applying the sample to a substrate, a surface of which is provided with a plurality of binding moieties each capable of binding specifically to the target cell so as to retain said target cells bound to said substrate surface, there being sufficient of said binding moieties to retain substantially all of the said target cells in the sample;

(b) washing the substrate and captured target cells to remove any extraneous non-specifically bound matter;

(c) lysing the retained target cells; and

(c) quantifying at least one component of the retained target cells to provide an indication of the number of retained target cells.

The term "binding specifically", is used to indicate that the binding moiety has the capacity to recognise and interact specifically with a particular molecular site or "marker" which is characteristic of the target cell.

There is commonly a low degree of affinity between any two molecules due to such non-covalent forces (such as electrostatic forces, hydrogen bonds, Van der Waals forces and hydrophobic forces) which is not restricted to a particular site on the molecules, and is largely independent of the identity of the molecules. This low degree of affinity can result in non-specific binding.

By contrast when two molecules bind specifically, the degree of affinity is much greater than such non-

specific binding interactions. In specific binding a particular site on each molecule interacts, the particular sites being structurally complementary, with the result that the capacity to form non-covalent bonds is increased. Examples of specific binding include antibody-antigen interactions and ligand-receptor interactions.

Preferably the sample is a fluid sample, such as a sample of a bodily fluid or derived from a bodily fluid. Preferably the sample is, or is derived from blood. For example, the fluid sample may be whole blood, concentrated red cells, platelet concentrates or plasma. The fluid sample may be a leucodepleted blood component.

The target cell may be any eukaryotic or prokaryotic cell. It is presently preferred that the target cell is a eukaryotic cell, for example a eukaryotic cell which may be found in a bodily fluid. Preferably the target cell is a cell which can be found in blood, for example monocytes, macrophages, neutrophils, eosinophils, basophils, B lymphocytes, T lymphocytes, natural killer cells, dendritic cells, erythrocytes or platelets. Preferably the target cell is a leucocyte.

Preferably the substrate is defined so that it is capable of discretely accommodating multiple samples. The solid substrate may be a microtitre plate, for example a 6-well, 12-well, 24-well, 48-well or a 96-well microtitre plate. Most preferably the solid substrate is a 96-well microtitre plate.

In the capturing step, the target cells bind specifically to the substrate such that the remainder of the sample can be removed, leaving the target cells bound (indirectly or directly) to the substrate. The substrate and the captured target cells are then washed

to remove any extraneous non-specifically bound matter.

To enable the substrate to capture target cells, a surface of the substrate is provided with a plurality of binding moieties, each of which is capable of binding specifically to the target cells. In this invention, the binding moiety is preferably an antibody to a cell-surface molecule or a ligand for a cell surface molecule such as an adhesion molecule.

The binding moiety which interacts may be bound to the substrate directly, for example by adsorbtion of the molecule comprising the binding moiety on to the substrate, or indirectly, for example by way of a linker molecule. If the binding moiety is an antibody, an example of a suitable linker molecule is streptavidin or similar molecule (such as ExtrAvidin which is commercially available from Sigma Chemicals).

In a preferred embodiment, the target cell is a leucocyte and the substrate is capable of binding to a leucocyte cell-surface antigen. For example, in this preferred embodiment the solid substrate may have bound to its surface antibodies against one or more of the following leucocyte cell surface antigens: CD3, CD5, CD13, CD14, CD15, CD16, CD19, CD20, CD34 and/or CD45. Other CD targets also exist. Preferably the solid substrate has bound to its surface antibodies against CD45 and CD15. More preferably, the solid substrate has bound to its surface antibodies against both CD45 and CD15. There are a number of other leucocyte cell surface antigens in addition to those listed above. The choice of cell surface antigen will depend on the target cell. For example, it is possible to target a particular leucocyte sub-population by using one or more leucocyte cell surface antigen(s) characteristic for that sub-population.

In another preferred embodiment of the invention,

the sample is a maternal blood sample of a rhesus-negative mother which is to be tested to quantitate the concentration of fetal, rhesus-positive red-blood cells in the maternal circulation. In this embodiment, the solid substrate may have bound to its surface a monoclonal antibody specific for D+ red blood cells, such as BRAD-3.

In the method of the invention, the cells are quantified by analysing a cellular component of the captured target cell. This component may be an intracellular component or a cell surface component. The cellular component of the target cells may be any molecule, the expression of which (in or on the target cell) is substantially uniform among the target cell population. In other words, the expression of intracellular or cell surface component of the target cells must be substantially proportional to the number of cells in the fluid sample.

The cellular component may be nucleic acid (DNA, rRNA, tRNA, mRNA), or an intracellular or cell surface protein, glycoprotein, carbohydrate or lipid. Preferably the intracellular component of the target cell is nucleic acid (DNA and RNA). In the embodiment of the invention which is for measuring the concentration of fetal, rhesus-positive red-blood cells in the maternal circulation, it is preferred that the cellular component quantified is haemoglobin.

After applying the sample to the substrate and the washing step, the cell membranes of the retained cells are disrupted or permeabilised prior to the quantification step. The cells may be lysed by conventional techniques such as incubation in a cell lysis solution, freeze-thaw, or sonication. Commonly used cell lysis solutions include solutions comprising a cell lysis agent such as a detergent or an enzyme,

and hypotonic solutions such as distilled water. There are also commercially available differential cell lysis solutions (for example, Optilyse C and DNA Prep LPR from Beckman Coulter). The choice of lysis means will depend on the cell population targeted.

The amount of the cellular component of the target cells may be analysed using a probe.

For example, such a probe may be capable of binding specifically to the cellular component of the target cells and capable of being detected when it is so bound. For example the probe may be protein such as an antibody or a ligand for a cell-surface or intracellular protein. Such a protein may be labelled, for example with a radiolabel, a fluorescent label or an enzyme label.

Alternatively, the probe may be capable of binding directly or indirectly to a cellular component of the target cells and being detected specifically when it is so bound. For example the probe may be a dye. Preferably the probe is a dye which is detectable when bound to nucleic acids. More preferably the probe is a dye which exhibits fluorescent enhancement when bound to cellular nucleic acids. Most preferably the probe is CyQUANT GR dye (Molecular Probes) or PicoGreen dye (Molecular Probes).

The bound probe may be detectable by any one of a number of known techniques such as fluorimetry, spectrophotometry, autoradiography or radioactive counting and chemiluminescence methods. Preferably the bound probe is detectable by fluorimetry, for example using a fluorescence microplate reader. When the probe is CyQUANT GR dye or PicoGreen dye, a fluorescent microplate reader is commonly used with filters appropriate for fluorescein (for example, about 480nm excitation and about 520nm emission maxima).

Alternatively, the cellular component may be analysed or detected directly without the use of a probe. For example, non-invasive techniques are known in the art by which an intracellular molecular component may be detected in situ in intact or lysed cells. Haemoglobin in erythrocytes, for example, may be detected by 3 wavelength spectrophotometry in a manner known *per se*. This method may be used in the aspect of the invention where the sample is a blood sample of a rhesus-negative mother which is to be tested to quantitate fetal rhesus-positive red-blood cells in the sample. More particularly, in the 3 wavelength spectrophotometry method mentioned above, after the lysis step, sample absorbance at wavelengths of 562nm, 578nm and 598nm is recorded. The concentration of haemoglobin is calculated as follows:

$$\frac{A_{562} - A_{598}}{2.25} = X$$

$$A_{562} - X = Y$$

$$(A_{578} - Y) x 155 = \text{haemoglobin in mg per dl}$$

The concentration of haemoglobin remaining can be used to calculate the proportion of fetal cells in the maternal blood sample, and consequently the volume of FMH.

According to a second aspect of the invention, there is provided a kit for quantifying the number of target cells in a sample using the method of the first aspect of the invention, which kit comprises:

(i) a substrate, the surface of which is provided with a plurality of binding moieties each capable of binding specifically to the target cell, or the means for making such a substrate.

In one embodiment, the kit of the second aspect of

the invention also comprises:

(ii) a probe capable of binding specifically to a cellular component of the target cells and capable of being detected when it is so bound, or a probe capable of binding to a cellular component of the target cells and capable of being detected specifically when it is so bound.

The kit may comprise a substrate which is ready-provided with binding moieties capable of binding specifically to the target cell.

Alternatively, the kit may comprise the means for making such a substrate. For example, the kit may comprise a substrate and a solution of molecules which comprise target-cell specific binding moieties in a form suitable for application to the substrate. The target cell-specific molecules may, for example, be absorbed on to the solid substrate by incubating the solution of the molecule on the solid substrate.

The kit may also comprise a cell lysis means, for example a cell lysis solution.

The kit may also comprise a means for detecting the probe. However, many of the detection means which may be used with the method of the first aspect of the invention (such as detection using a fluorimetric plate reader) are unsuitable for incorporation into kit form.

Thus, the present invention discloses a method for quantifying cells which can be carried out in a microplate and which lends itself to simultaneous automated cell counting for multiple samples.

In one embodiment of the invention, there is provided a technique for counting leucocytes in solution which involves capture of the leucocytes on a substrate, and staining the nucleic acid with a quantitative fluorescent stain.

In another embodiment, there is provided a method for quantitating fetal rhesus-positive red-blood cells in a blood sample of a rhesus-negative mother which involves capture of the foetal rhesus-positive red-blood cells on a substrate, and measuring the amount of haemoglobin in the captured cells from which a measure of the proportion of fetal cells in the maternal blood sample can be determined, and hence the volume of FMH.

The method is capable of consistently detecting leucocytes at concentrations down to concentrations of the order of 1 leucocyte μL^{-1} in whole blood, concentrated red cells, platelet concentrates and plasma. Detection is generally linear throughout the range 0.1-100 leucocytes μL^{-1}. By carrying out the technique in a standard microtitre plate, it is possible to analyse up to 96 samples simultaneously. The method is inexpensive, quick, accurate and reproducible.

The method can be used for many cell quantification applications. It is generally applicable to any circumstance when it is necessary to quantitate specific cell types which are present in low numbers in mixed populations (for example, cell types which are present in low numbers in body fluids). Examples of such circumstances include detection of CD34+ haemopoietic stem cells, analysis of fetal rhesus-positive red-blood cells in rhesus-negative mothers, analysis of cancer cells in leukaemia and detection of cells in cerebrospinal fluid (CSF).

For a better understanding of the present invention, and to show how the same may be put into effect, reference will now be made, by way of example only, to the following examples, in which reference is made to Figures 1 to 5, as follows.

Figure 1: Fluorescent intensity against leucocyte

concentration in platelets.

Figure 2: Fluorescent intensity against leucocyte concentration in SAG-M red cells.

Figure 3: Fluorescent intensity against leucocyte concentration in whole blood.

Figure 4: Fluorescent intensity against leucocyte concentration in leucodepleted platelet concentrate.

Figure 5: Fluorescent intensity against leucocyte concentration in unfixed and fixed whole blood samples.

EXAMPLES

Example 1

Quantitation of leucocyte concentration in various samples

Preparation and analysis of blood components

The microplate assay used buffy coat depleted SAG-M red cells, plasma and platelets. Platelets were leucodepleted by in process filtration, with an in-line ATSBC-1 Autostop (Pall) filter. Plasma was leucodepleted using the Gelman filter from a PathInact Methylene Blue kit (Baxter). SAG-M red cells were leucodepleted using a BP4 (Pall) filter. Whole blood was leucodepleted using an integral RS2000 filter (Asahi). The leucocyte content of all prefiltration samples, except for plasma, was determined using a Sysmex haematology analyser. All filtered products, and unfiltered plasma, were analysed by flow cytometry as described previously (Rider et al., 1996 as above).

Microplate fluorescence assay

ExtrAvidin (Sigma) was prepared to 20μg mL^{-1} in bicarbonate buffer (pH 9.6) (3g of $NaHCO_3$, 1.5g of Na_2CO_3, made up to 500ml with water) and 50μL was added to each well of Immulon 4 (Dynex Technologies, Billingshurst, UK) 96-well microplates. Plates were

incubated overnight at 4°C before manual aspiration. This was followed by the addition of 50μL of an antibody cocktail (20μg mL^{-1} of biotinylated anti-CD45 and 10μg mL^{-1} of biotinylated anti-CD15 in PBS pH 7.4 + 1% human serum albumin) to each well. Plates were incubated with agitation at 37°C for 2 hours and manually aspirated as before. A 200μL volume of sample was then added to all wells. The sample types investigated were:

- Whole Blood
- Plasma
- SAG-M red cells
- Platelets

Calibrators were prepared by dilution of component with PBS or with the leucodepleted equivalent component to give a series of known leucocyte concentrations. To test the agreement of the assay with current routine tests, blood product samples already analysed by flow cytometry in routine Product Testing were used. In all nine apheresis platelet samples, one haemonetics platelet sample and ten citrate-phosphate-dextrose leucodepleted red blood cell (CPD-LD-RBC) samples were tested[1].

Plates, loaded with sample, were incubated with agitation for 2 hours at 37°C, before being either just blotted or blotted and gently washed with PBS. Plates were frozen at -70°C for at least one hour. After defrosting, "blank plate" readings without the addition of nucleic acid stain were taken, using a fluorescent plate reader with excitation at 485nm and emission at

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[1] You ask whether the results are shown. It looks to me as though they are not. Do you have them? Are they important?

530nm. CyQUANT (Molecular Probes Europe, Leiden, Netherlands) working reagent containing lysis buffer and DNA stain was prepared according to the manufacturer's instructions and 200μL added to each well. The plate was incubated in the dark for 5 minutes before being analysed on the plate reader as above. Longer incubation periods (for example of about 1 hour) may alternatively be used. All fluorescence readings were corrected by subtraction of fluorescence blanks. An alternative approach is to use a well containing 200μL of CyQUANT as the blank and then the value obtained is used to correct all other fluorescence readings.

Results

Linear dose responses were observed with spiked platelets, red blood cells and whole blood (figures 1-3). Similarly, plasma gave a dose response but this was less easy to investigate as the leucocyte concentrations in this product are inherently low (0-20 cells μL^{-1}).

The effect of washing the plates after sample removal was sample type dependent. With platelets and plasma, plates emptied of sample but not washed before freezing had greater (3-4x) fluorescent intensities when stained with CyQUANT than washed plates. However with spiked red cells and whole blood, readings from unwashed plates showed no dose response and had fluorescent intensities several times lower than corresponding results from washed plates due to fluorescence quenching by haemoglobin.

To assess the effectiveness of the fluorescence assay as a standard quality control test, twenty assorted blood products were analysed. Assays were performed in duplicate using the standard protocol, with washing. Red cell and platelet samples were

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analysed on the same plate. All of the products were leucodepleted to 1-4 leucocytes μL^{-1}, as determined by flow cytometry, except one platelet sample, which contained 110 leucocytes μL^{-1}. Mean fluorescence intensities for all samples were between 7656 and 5025 units, except the leucodepleted sample, which had a mean intensity of 17033 units. Percentage error of duplicates ranged from 2.1% to 25.7%.

In the assay shown in Figure 4, a 96-well Immulon4 plate was prepared using the standard protocol. Leucodepleted platelet concentrate was spiked with leucocytes isolated from a whole blood sample (7ml EDTA) using histopaque. A range of leucocyte concentrations from 6 to 200 leucocytes μL^{-1} were tested. Following a 2 hour incubation and freeze-thaw step, plates were stained with CyQUANT and read at excitation 485/20, emission 530/25 and sensitivity 64. This assay showed that all leucocyte concentrations down to 6 leucocytes μL^{-1} gave above background readings.

In the assay shown in Figure 5, a 96-well Immulon4 plate was prepared using the standard protocol. Two whole blood samples (7ml EDTA) were obtained. 70μl of fixative solution (Transfix-NEQAS) was added to one sample. The samples were diluted with PBS to give a range of leucocyte concentrations from 3 to 4000 leucocytes μL^{-1}. Following a 2-hour incubation and freeze-thaw step, plates were stained with CyQUANT and read at excitation 485/20, emission 530/25 and sensitivity 60. This assay showed that leucocyte concentrations down to 3 leucocytes μL^{-1} in samples, both with and without fixative, gave above background readings.

Example 2

A biotinylated monoclonal antibody specific for

fetal D+ RBCs, such as ERAD-3, is adhered to the wells of a 96 well plate using ExtrAvidin. Duplicate 100μL samples of maternal blood are added to the microplate. The plate is incubated, with gentle shaking, at room temperature for 2 hours to facilitate binding of the antibody to any fetal D+ cells present. Following the incubation period, the samples are washed to remove any unbound RBCs. The captured fetal RBCs are lysed and sample absorbance at wavelengths of at 562nm, 578nm and 598nm is recorded. The concentration of haemoglobin is calculated as follows:

$$\frac{A_{562} - A_{598}}{2.25} = X$$

$$A_{562} - X = Y$$

$$(A_{578} - Y)x155 = \text{ haemoglobin in mg per dl}$$

The concentration of haemoglobin remaining can be used to calculate the proportion of fetal cells in the maternal blood sample, and consequently the volume of FMH.

Results

Following a 4ml FMH, 0.2% of the maternal RBC volume in a D- mother will be D+ fetal RBCs. A 100μL sample of blood contains approximately $4x10^8$ red blood cells. Therefore, 100μL of maternal blood, following a 4ml FMH, will contain approximately $8x10^5$ fetal D+ RBCs. Initial experiments have shown that the microplate assay can accurately detect RBCs at concentrations ranging from $4x10^5$ to $4x10^7$ RBCs per 100μL. This represents a potential FMH range from 2mL to 200mL.

In conclusion, the microplate assay represents a simple, high throughput alternative to the Kleihauer-Betke acid elution technique or to flow cytometry, for quantitating FMH.

Although less preferred, it will be appreciated

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that, instead of lysing the retained cells in step (c), the cells may be quantified by analysing a cellular component of the captured target cell which is a cell surface component, the expression of which (on the target cell) is substantially uniform among the target cell population, in other words that the expression of the cell surface component of the target cells is substantially proportional to the number of cells in the fluid sample.

CLAIMS

1. A method for quantifying the number of target cells in a sample which comprises;

(a) applying the sample to a substrate, a surface of which is provided with a plurality of binding moieties each capable of binding specifically to the target cell so as to retain said target cells bound to said substrate surface, there being sufficient of said binding moieties to retain substantially all of the said target cells in the sample;

(b) washing the substrate and captured target cells to remove any extraneous non-specifically bound matter;

(c) lysing the retained target cells; and

(c) quantifying at least one component of the retained target cells to provide an indication of the number of retained target cells.

2. A method according to claim 1, wherein the sample is a leucodepleted blood component and the target cell is a leucocyte.

3. A method according to claim 1 or 2, wherein the binding moieties are the antigen binding-sites of anti-CD45 antibodies and anti-CD15 antibodies.

4. A method according to any preceding claim, wherein the cellular component is nucleic acid.

5. A method according to any preceding claim, wherein the cellular component is quantified using a probe.

6. A method according to claim 4, wherein the nucleic acid is quantified using a probe which is capable of binding nucleic acids and being detected specifically when it is so bound.

7. A method according to claim 6, wherein the probe is CyQUANT GR dye.

8. A method according to claim 6, wherein the probe is PicoGreen dye.

9. A method according to claim 1, wherein the sample is a maternal blood sample of a rhesus-negative mother which is to be tested to quantitate fetal rhesus-positive red-blood cells in the sample.

10. A method according to claim 9, wherein fetal red blood cell haemoglobin is quantitated in the lysed cell sample.

11. A kit for use in quantifying the number of target cells in a sample using a method according to any preceding claim, which kit comprises a substrate, the surface of which is provided with a plurality of binding moieties each capable of binding specifically to the target cell, or the means for making such a substrate.

12. A kit according to claim 9, which also comprises a probe capable of binding specifically to a cellular component of the target cells and capable of being detected when it is so bound, or a probe capable of binding to a cellular component of the target cells and capable of being detected specifically when it is so bound.

Fig. 1

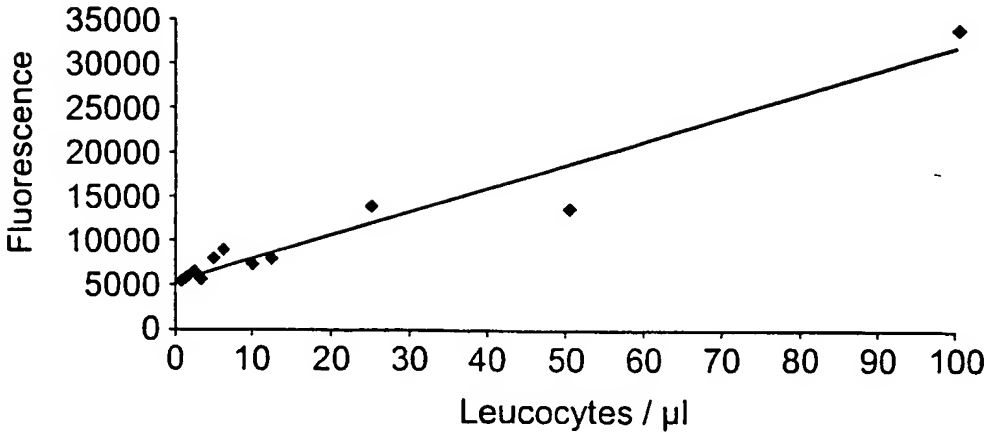

Fig. 2

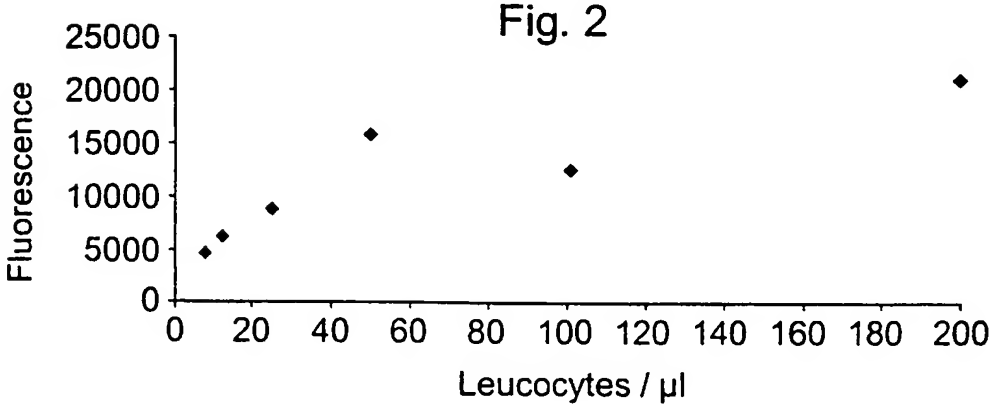

Fig. 3

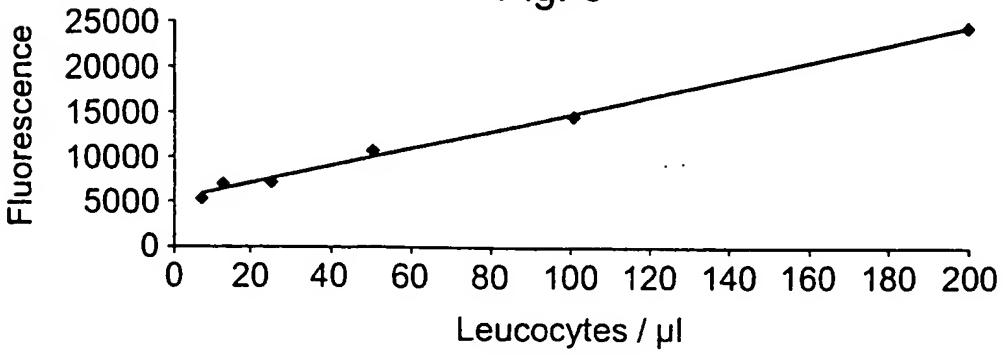

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Fig. 4

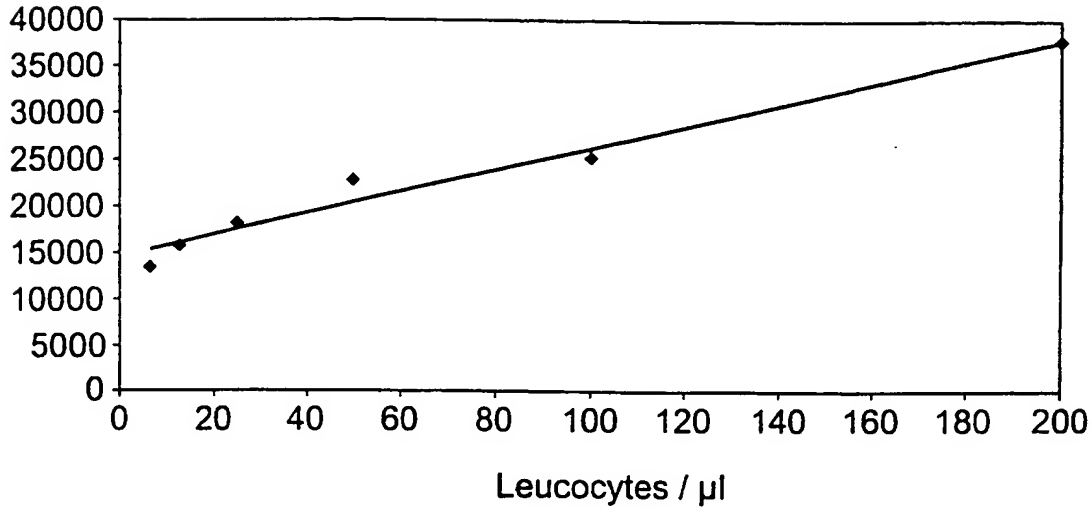
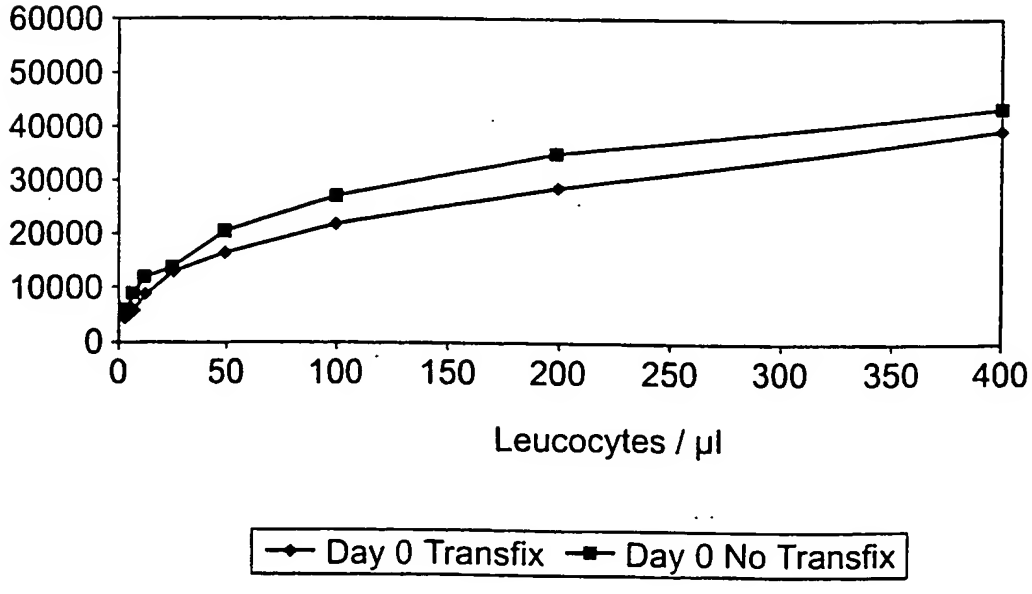


Fig. 5



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INTERNATIONAL SEARCH REPORT

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A. CLASSIFICATION OF SUBJECT MATTER
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According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, PAJ, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	EP 0 311 492 A (SANOFI SA) 12 April 1989 (1989-04-12)	1-10
X	claims	11,12
Y	PATENT ABSTRACTS OF JAPAN vol. 017, no. 654 (P-1653), 3 December 1993 (1993-12-03) & JP 05 215750 A (IDEMITSU PETROCHEM CO LTD), 24 August 1993 (1993-08-24) abstract	1-12

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
25 October 2000	06/11/2000

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	GONCALVES M L F C

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INTERNATIONAL SEARCH REPORT

Interna' al Application No
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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	DATABASE WPI 38 Derwent Publications Ltd., London, GB; AN 1993-300220 XP002151026 "Immunoassay method-by fixing antibody to surface antigen of analyte cell on baseplate, contacting sample containing analyte cell , etc" & JP 05 215750 A (IDEMITSU PETROCHEM CO), 24 August 1993 (1993-08-24) abstract	1-12
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Y,P	DATABASE WPI 47 Derwent Publications Ltd., London, GB; AN 1999-561701 XP002151027 "Immunological method for counting Leucocytes with a miniaturizable counter, at low cost, with accuracy, without interfrence from other nucleated cells, precipitated fibrin or the skill of operator" & JP 11 258231 A (KYOTO DAIICHIKAGAKU CO LTD), 24 September 1999 (1999-09-24) abstract	1-12
A	SCHEER W D: "THE DETECTION OF LEUKOCYTE ESTERASE ACTIVITY IN URINE WITH A NEW REAGENT STRIP" AMERICAN JOURNAL OF CLINICAL PATHOLOGY,PHILADELPHIA, PA,US, 1987, pages 86-93, XP000918195 ISSN: 0002-9173 see summary	1-12
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A	PICARD F ET AL: "PRELIMINARY EVALUATION OF THE NEW HEMATOLOGY ANALYZER COULTER GEN.S IN A UNIVERSITY HOSPITAL" CLINICAL CHEMISTRY AND LABORATORY MEDICINE,WALTER DE GRUYTER UND CO,DE, vol. 37, no. 6, 1999, pages 681-686, XP000918440 ISSN: 1434-6621 see summary ----	1-12

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